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GENETIC AND PHYSIOLOGICAL CONTROL OF PROTECTIVE ANTIGEN SYNTHESIS BY BACILLUS ANTHRACIS

ANNUAL PROGRESS REPORT

CURTIS B. THORNE

DECEMBER 1981

Supported by

U. S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701

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University of Massachusetts
Amherst, Massachusetts 01003

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The primary objective of the research is to gain information and to develop genetic systems that will contribute to the development of an improved vaccine for anthrax. Genetic and physiological factors controlling the synthesis and accumulation of protective antigen as well as the two other components of anthrax toxin are being investigated. Attention was concentrated on the following areas during the past year: Spontaneous variation in B. anthracis; Is the presence of a plasmid or prophage related to toxin synthesis; and Genetic exchange systems, isolation of mutants, and chromosomal mapping.

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20. Abstract (Continued)

Phage CP-51 is an effective generalized transducing phage for B. anthracis, and it is being used for chromosomal mapping. For this purpose a number of auxotrophic mutants of the Weybridge strain have been isolated and characterized.

Phage CP-51 is effective also in transferring the tetracycline resistance plasmid, pBC16, among strains of B. cereus, B. thuringiensis, and B. anthracis. pBC16 is being used as a plasmid marker in the Weybridge strain.

Variants of the Weybridge strain cured of a large plasmid failed to produce detectable amounts of protective antigen. Such variants retained the original auxotrophic marker of the parent strain, but in addition they demonstrated new nutritional requirements. It is unclear whether the additional nutritional requirements resulted from loss of the plasmid or whether they reflect new chromosomal mutations induced by the curing agents.

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Summary

This is a progress report (annual report) of research being carried out with Bacillus anthracis. The primary objective of the research is to gain information and to develop genetic systems that will contribute to the development of an improved vaccine for anthrax. Genetic and physiological factors controlling the synthesis and accumulation of protective antigen as well as the two other components of anthrax toxin are being investigated. Attention was concentrated on the following areas during the past year: Spontaneous variation in B. anthracis; Is the presence of a plasmid or prophage related to toxin synthesis; and Genetic exchange systems, isolation of mutants, and chromosomal mapping.

The phenomenon of spontaneous variation at high frequencies remains one of the most perplexing problems in research on B. anthracis. The fact that the high frequency of spontaneous mutations does not apply to all characteristics renders unlikely the hypothesis that a mutator gene may be involved. The finding that strains cured of the B. anthracis plasmid have additional nutritional requirements compared to those of the parent strain may offer some insight into the problem. Another observation which may suggest experimental approaches is that of the correlation between colonial morphology of variants and the frequency with which they sporulate.

Weybridge A, a variant isolated from the wild-type Weybridge strain, grows well on minimal O medium which contains, in addition to glucose, salts and glutamic acid, only five amino acids and thiamine. Minimal O therefore serves well as a minimal medium for the isolation of a wide variety of auxotrophic mutants. A number of auxotrophs have been isolated. Among them are mutants requiring valine, nicotinic acid, leucine, leucine plus valine, phenylalanine, adenine, uracil, and arginine plus uracil(pyrA).

Phage CP-51 has been shown to be an effective generalized transducing phage for the Weybridge strain, and it is being used for chromosomal mapping by cotransduction of linked markers. Thus far two linkage groups have been established; one involves phenylalanine and nicotinic acid mutations which are cotransduced at a frequency of 96%, and the other involves two pyrimidine mutations which are cotransduced at a frequency of 91%.

Phage CP-51 is also effective in transferring a tetracycline resistance plasmid, pBC16, from B. cereus to the Weybridge strain and strains of B. thuringiensis. We have used pBC16 as a plasmid marker in the Weybridge strain.

The Weybridge strain carries a plasmid of high molecular weight as shown by gel electrophoresis. In experiments designed to cure the Weybridge strain of the large plasmid we used an auxotrophic mutant, pyrA, which also carried plasmid pBC16. The mutant was grown in broth containing a mixture of known curing agents, i.e., novobiocin, acridine orange, ethidium bromide, and sodium dodecylsulfate. Surviving cells were screened for loss of the Tet^R plasmid, and tetracycyline-sensitive cells were then tested for simultaneous loss of the B. anthracis plasmid. About 10% of cells cured of the Tet^R plasmid had simultaneously lost the B. anthracis plasmid. Cured strains failed to produce any protective antigen detectable by the agar diffusion method of assay. Although strains cured of the B. anthracis plasmid retained their original auxotrophic marker, they had additional growth requirements not demonstrated by the parent strain. It is presently unclear whether the additional nutritional requirements resulted from loss of the plasmid or whether they reflect new chromosomal mutations induced by the curing agents.

Protoplasts of Weybridge cells carrying the tetracycline resistance plasmid, pBC16, were fused with protoplasts of B. subtilis Trp^- Str^R in the presence of polyethyleneglycol. Selection on regeneration medium was for fusons that were Tet^R and Str^R. These in turn were shown to have retained the Trp^- marker of the parent B. subtilis. Analysis of their DNA confirmed the presence of plasmid pBC16. We are now testing additional fusons looking for ones that have gained the large B. anthracis plasmid in addition to pBC16.

When grown under appropriate conditions cultures of the Weybridge strain lysed following the addition of mitomycin C. Examination of concentrated lysates by electron microscopy revealed particles resembling phage heads. The Weybridge strain also produces a bacteriocin-like activity which is active on only a few strains of B. cereus and B. thuringiensis. The relationship, if any, of the phage-like particles found in mitomycin C-induced lysates to the bacteriocin-like activity is not clear.

Foreward

Citations of trade names in this report does not constitute an official Department of Army endorsement or approval of the use of such items.

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This is the second annual report submitted under contract No. DAMD17-80-C-0099. Research on the contract began July 1, 1980 and the first annual report dated December 1980 covered the first six months of the project. The contract was extended for a second year beginning July 1, 1981. This second annual report, which covers the period January 1, 1981 to December 31, 1981, is submitted in conjunction with an application for a second extension of the contract. The research proposal submitted for the first extension of the contract proposed the following areas of investigation: I. Spontaneous variation in B. anthracis; II. Is the presence of a plasmid or prophage related to toxin synthesis; III. Genetic exchange systems, isolation of mutants, and chromosomal mapping; and IV. Physiological and metabolic factors affecting protective antigen synthesis and accumulation. During the past year our research has touched on all the areas mentioned and we have made some progress in each of them. In the following report each of the areas is discussed individually following a general description of materials and methods. Specific procedures which themselves are results of the research are described as appropriate under individual sections.

MATERIALS AND METHODS

Organisms. The Weybridge (1) strain of B. anthracis was obtained from the Microbiological Research Establishment, Porton, England, in 1957. It was isolated by Sterne (2) and used by the Ministry of Agriculture, Fisheries, and Food (Weybridge, England) as a living spore vaccine. Strain V770 was obtained from Anna Johnson, USAMRIID, Fort Detrick, Frederick, Maryland. B. cereus GP7 was obtained from W. Goebel. The other strains of B. subtilis, B. cereus, and B. thuringiensis were from my stock collection.

Media. For convenience to the reader compositions of the various media mentioned in this report are given below in detail. All amounts are for one liter, final volume. For preparations of solid medium, 15 grams of Difco agar were added per liter of the corresponding broth.

NBY broth: Difco nutrient broth, 8 g; Difco yeast extract, 3g.

Nutrient broth: Difco nutrient broth, 8 g.

L broth: Difco tryptone, 10 g; Difco yeast extract, 5 g; NaCl, 10 g. pH adjusted to 7.0 with NaOH.

PA (phage assay) broth: Difco nutrient broth, 8 g; NaCl, 5 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $MnSO_4 \cdot H_2O$, 0.05 g; $CaCl_2 \cdot 2H_2O$, 0.15 g; pH adjusted to 6.0 with HCl.

Brain heart infusion broth (BHI): Difco brain heartinfusion broth, 37 g.

Minimal I: $(NH_4)_2SO_4$, 2 g; KH_2PO_4, 6 g; K_2HPO_4, 14 g; sodium citrate, 1 g; glucose, 5 g; L-glutamic acid, 2 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $FeCl_3 \cdot 6H_2O$, 0.04 g; $MnSO_4 \cdot H_2O$, 0.00025 g; pH adjusted to 7.0 with NaOH. The glucose and $FeCl_3$ were sterilized separately.

Minimal IC: Minimal I with 5 g of Difco vitamin-free casamino acids.

Minimal M: To minimal I was added 10 mg of thiamine hydrochloride, 200 mg of glycine, and 40 mg of L-methionine and L-proline.

Minimal O: To minimal I was added 10 mg of thiamine hydrochloride, 200 mg of glycine, and 40 mg of L-methionine, L-serine, L-threonine, and L-proline.

Antisera. The antisera were supplied by Anna Johnson of USAMRIID. They were prepared in a horse and a burro by injecting spores of the Sterne strain.

Antigen assays. These were carried out by the agar diffusion method as outlined by Thorne and Belton (1).

Phage CP-51 propagation and assay. The methods described by Thorne (3) were followed. The indicator for assay of CP-51 was B. cereus NRRL 569.

RESULTS AND DISCUSSION

I. Spontaneous variation in B. anthracis.

The phenomenon of spontaneous variation at high frequencies remains one of the most perplexing problems we have encountered in our work on B. anthracis, and it is probably one of the most important. For consistent and dependable production of protective antigen it would be desirable to have a stable strain. A case in point which illustrates the nutritional variability among strains is the recent finding in my laboratory that a wild-type Sterne strain obtained from Dr. G. Knudsen at USAMRIID has growth requirements different from those of the wild-type Weybridge strain in use in my laboratory. Presumably the two strains came from the same original source.

As reported in the Annual Report of December 1980 we isolated two variants with respect to growth requirements from the wild-type Weybridge strain. Weybridge A grows quite well on minimal O agar which contains the amino acids glutamic acid, glycine, methionine, serine, threonine, and proline. It grows less well, but adequately, on minimal M, which contains only four amino acids, glutamic acid, glycine, methionine, and proline. Weybridge B is nutritionally more complex. We have not taken the time to determine its absolute requirements, but we have shown that it will grow, although not as well as Weybridge A, if branched chain amino acids are added to minimal O.

Compared to my wild-type Weybridge A strain the wild-type Sterne strain obtained from Dr. Knudsen grows very poorly on minimal O. However, it grows very well on minimal O supplemented with cystine. It resembles our variant, Weybridge B, in that occasional colonies in the population grow well on unsupplemented minimal O. Presumably these are variants similar to Weybridge A. (However, it should be made clear that my Weybridge B variant will not grow well on minimal O supplemented with cystine).

The complexity of nutritional requirements and the antagonistic relationships among various amino acids are greater in B. anthracis than in any other Bacillus species I have studied, although strains of B. thuringiensis resemble B. anthracis in this respect. The finding described below that strains

cured of the toxin plasmid have additional nutritional requirements compared to those of the parent strains may offer some insight into the spontaneous variation problem. One possibility is that the plasmid may carry structural or regulatory genes involved in growth and metabolism. Another possibility is that certain genes may shift from chromosome to plasmid and vice versa. Thus, we are examining strains cured of the toxin plasmid to (1) determine their nutritional requirements and (2) determine whether they differ from uncured strains with respect to stability and spontaneous variation. Such investigations are in progress in conjunction with our other studies on variation.

One hypothesis considered earlier was that B. anthracis might carry a mutator gene analogous to those reported for other organisms (4). This now seems unlikely since observations have indicated that the high frequency of spontaneous variation does not apply to all genes. For example, spontaneous mutations to streptomycin resistance, tetracycline resistance (chromosomal), or temperature sensitivity apparently do not occur at unusually high frequencies.

We have observed that often there is a correlation between the colonial morphology of variants and the frequency at which they sporulate. Also we have observed in variants a correlation between the ability to grow on a less complex medium and the loss of sporulation ability. It is not clear why asporogenous mutants should have fewer nutritional requirements than the wild-type strain from which they arose. However, these observations suggest that much of the spontaneous variation in cultures of B. anthracis may be closely related to changes in sporulation characteristics. Very preliminary observations in my laboratory suggest that strains cured of the toxin plasmid may sporulate faster and/or at a higher frequency than uncured strains. We will examine this in considerable detail to determine whether the toxin plasmid has any influence on sporulation. Lovett (5) reported enhanced sporulation of plasmid-negative variants of Bacillus pumilus.

II. Is the presence of a plasmid or prophage related to toxin synthesis?

(1) Isolation of plasmid-negative variants. The Annual Report of December 1980 discussed the difficulty we experienced in firmly establishing

that cells of the Weybridge strain carry a plasmid of high molecular weight. The experiments reported there had been done using the Currier and Nester (6) procedure for isolation of plasmid DNA. After that report was written, we discontinuted using that procedure in favor of the procedures of Kado and Lui (7) and White and Nester (8). With these procedures the large plasmid was reproducibly demonstrated and we therefore began testing procedures for isolating variants cured of the plasmid.

At about the same time the group at USAMRIID reported their success in curing B. anthracis of the large plasmid and demonstrating that cured strains did not produce toxin, we also obtained cured strains by a different approach. In our approach we looked for simultaneous curing of the tetracycline resistance plasmid, pBC16, and the B. anthracis plasmid. We used an auxotrophic mutant of the Weybridge A strain, M18 (pyrA , requires both arginine and uracil for growth) so that contaminants would not be mistaken for cured strains. In addition the mutant carried plasmid pBC16, encoding tetracycline resistance, which was introduced into the Weybridge strain by transduction with CP-51 (see below).

The mutant was grown in L broth on the shaker at 37°C overnight and 2.5 ml was transferred to 25 ml of fresh L-broth. Incubation was continued with shaking at 37°C and after three hours the following were added to give the final concentrations indicated: acridine orange, 20 µg/ml; sodium dodecylsulfate, 10 µg/ml; ethidium bromide, 1 µg/ml; novobiocin, 0.1 µg/ml. Incubation at 37°C was continued for 16 hours at which time cells from 15 ml of the culture were collected by centrifugation and added to 25 ml of fresh L broth. Following incubation for another 6 hours on the shaker at 37°C the culture was plated on L agar to obtain isolated colonies. A number of the resulting colonies were then picked to L agar and to L agar containing 40 µg of tetracycline per ml to screen for cells cured of the tetracycline resistance plasmid. Approximately 10% of the colonies were Tet^S. When the Tet^S colonies were examined for presence of plasmid by gel electrophoresis, all were free of pBC16 and about 10% were also free of the large B. anthracis plasmid.

Two of the variants cured of the B. anthracis plasmid were tested for protective antigen production in the casamino acids medium (1) and no antigen was detected by the agar diffusion method of assay. In addition colonies growing near wells of antiserum on agar plates of casamino acids medium produced no visible lines of precipitation. In both of these tests the parent strain, Weybridge A M18, gave positive results as normally obtained.

The cured strains retained the auxotrophic marker of the parent M18 as evidenced by their growth response to uracil on casamino acids medium (Minimal IC). However, they had acquired an additional growth requirement as evidenced by the fact that they would not grow on minimal O supplemented with arginine and uracil. In this connection it is interesting, and perhaps significant, that a cured strain isolated by Dr. G. Knudsen at USAMRIID and sent to me for testing also had different growth requirements from its parent strain. As mentioned above, Knudsen's wild-type strain grew on minimal O medium supplemented with cystine. However, his cured strain did not respond to cystine.

The new growth requirements of the cured strains have not yet been identified; nor is it known whether all cured strains have the same requirements. It is conceivable that some genes involved in growth and metabolism are carried on the large plasmid. Another possibility is that the new growth requirements reflect new chromosomal mutations. The fact that the agents used to obtain the cured strains are also mutagenic agents strengthens this latter possibility.

(2) Defective phage and bacteriocin activity. Experiments have continued on investigation of defective phage reported by Nagy and Ivanovics (9) to be carried by Sterne-type strains. When grown under appropriate conditions, cultures of the Weybridge strain lysed following the addition of mitomycin C (0.5-1.0 µg/ml). Particles present in lysates were concentrated by high-speed centrifugation and resuspension of the pellet in a small volume of buffer. Examination of concentrated lysates by electron microscopy revealed particles resembling phage heads. Such particles were not found in concentrated filtrates from uninduced cultures.

Lysates were tested for activity in lawns of 16 strains of B. cereus and 37 strains of B. thuringiensis. Only one strain of B. cereus and three strains of B. thuringiensis showed any sort of sensitivity to the lysates. Further tests, however, indicate that the sensitivity of those strains is not to the defective phage but to a bacteriocin-like activity which the Weybridge strain produces. The bacteriocin is best demonstrated by replica plating from lawns of sensitive strains to nutrient agar plates containing colonies of the Weybridge strain. The most sensitive strains thus far tested are B. cereus 9620 and B. thuringiensis 4058.

The relationship, if any, of the phage-like particles found in mitomycin C-induced lysates to the bacteriocin-like activity is unclear. The two may be completely unrelated. Presumably the bacteriocin activity is not associated with the toxin plasmid; strains cured of the toxin plasmid remained bacteriocin-positive. Strains cured of the toxin plasmid have not yet been tested to determine whether they still carry defective phage.

III. Genetic exchange systems, isolation of mutants, and chromosomal mapping.

(1) Transduction of chromosomal markers with phage CP-51. Several years ago I isolated a phage, designated CP-51 (3), which is active in generalized transduction of B. cereus, B. thuringiensis, and B. anthracis. We have used the phage for some mapping studies in B. cereus and B. thuringiensis, but its virulent nature causes problems in recovery and analysis of transductants. At the time I isolated CP-51 we did not use it in extensive studies with B. anthracis; we merely demonstrated that it does carry out transduction in the Weybridge strain (10).

In investigations under the current contract on B. anthracis genetics we have taken a closer look at transduction of the Weybridge strain with CP-51. Fortunately, we have found that the phage is much less lytic for B. anthracis than it is for B. cereus and B. thuringiensis, and problems in recovering transductants and analyzing their genetic constitution are minimal. Thus, we have been able to begin chromosomal mapping studies.

Table 1 gives some transduction data obtained with mutants of the Weybridge strain. The two pyrimidine mutations were cotransduced at a

Table 1

Transduction of mutants of Weybridge A strain with phage CP-51*

Donor	Recipient	PFU/plate	Transductants per plate	Frequency of transduction	No. doubles / No. tested	Cotransduction (%)
M11 Leu$^-$	M2 Trp$^-$	3.5×10^7	280	8×10^{-6}	0/174	0
M14 Phe$^-$	M2 Trp$^-$	3.7×10^7	266	7.2×10^{-6}	0/56	0
M14 Phe$^-$	M4 Nic$^-$	3.7×10^7	200	5.4×10^{-6}	162/168	96.4
M23 Ura$^-$	M18 Car$^-$	1.7×10^7	148	8.7×10^{-6}	101/111	91.0

*Cells (0.1 ml) and 0.1 ml of phage containing the given number of PFU were spread on minimal medium supplemented with the requirement of the donor, i.e., leucine, phenylalanine, or uracil plus arginine. Transductants were picked to minimal medium and minimal medium supplemented with donor requirement to test for cotransduction.

frequency of 91%. (The Car^- mutant requires both arginine and uracil and is probably defective in carbamoylphosphate synthetase. In B. subtilis mutants of this phenotype are given the genotypic designation, pyrA). The phe and nic mutations were cotransduced at a frequency of 96.4%. Both of these linkages are analogous to linkages in B. subtilis and B. thuringiensis. In B. thuringiensis leu and trp mutations are cotransducible with CP-51 but in B. subtilis they are not cotransducible with a phage larger than CP-51. It may be that in this region of the chromosome the B. anthracis map will turn out to resemble the map of B. subtilis more than that of B. thuringiensis.

(2) Transduction of plasmid pBC16 by CP-51. It seemed desirable to have a drug resistance plasmid in the Weybridge strain to use as a plasmid marker. If such a plasmid were expressed efficiently in the Weybridge strain, and if it persisted through many transfers of the culture, it would be useful in experiments on "co-curing," i.e., we could select variants cured of the drug resistance plasmid and test them for simultaneous loss of the anthrax toxin plasmid. Similarly such a plasmid would be useful in (1) working out a plasmid DNA transformation system for B. anthracis and (2) testing for simultaneous transfer of the resistance plasmid and the toxin plasmid.

pBC16 is a plasmid that carries genes for tetracycline resistance. It has a molecular weight of about 2×10^6 and it was found in B. cereus strain GP7 by Bernhard, et al. (11) in Germany. Tests for transfer of the plasmid showed that CP-51 is capable of transferring it from B. cereus to B. thuringiensis and B. anthracis as well as among B. anthracis and strains of B. thuringiensis.

Table 2 shows data obtained from transduction tests in which CP-51 was propagated on B. cereus GP7 and used to transduce Weybridge A M18 and two strains of B. thuringiensis to tetracycline resistance.

Table 2

Transduction of plasmid pBC16 from B. cereus GP7 to B. thuringiensis and B. anthracis*

Recipient	Transduction Mixture Cells	Phage	Tet^R colonies/ml
B. thuringiensis 4042B M45 Trp^-	1 ml	0.5 ml	35
B. thuringiensis 4060C M473 Ind^-	1 ml	0.5 ml	5
B. anthracis Weybridge A M18 (pyrA)	1 ml	0.5 ml	5

*Recipient cultures were grown in 25 ml of BHI broth containing 0.5% glycerol in a 250-ml flask. After 16 hours, 2.5 ml were transferred to fresh medium and incubated for 4.5 hours. One ml of cells (approximately 4×10^8), 0.5 ml of phage (3.5×10^9 PFU) grown on B. cereus GP7, and 0.5 ml of PA broth were incubated together in 20-mm tubes on a shaker at 37°C. After 2 hours, 0.1 ml of CP-51 antiserum (diluted 1 to 10) was added to each tube and incubation was continued 90 min. Samples (0.2 ml) were plated along with 0.1 ml of phage antiserum (diluted 1 to 10) on L agar containing 25 µg of tetracycline per ml. Colonies were scored after 24 hours at 37°C. No Tet^R colonies were obtained from control mixtures in which 0.5 ml of PA broth was substitutued for the phage.

The frequency of transfer was low (3×10^{-9} to 2×10^{-8}) but adequate for recovery of Tet^R transductants. Analysis by gel electrophoresis of DNA from representative transductants of each recipient showed that plasmid pBC16 was present in each of them.

The data in Table 3 show that CP-51 could transfer the plasmid among strains quite efficiently. Frequencies as high as 1.5×10^{-7} Tet^R transductants per PFU were obtained.

Table 3

Transfer of pBC16 among B. anthracis and strains of B. thuringiensis by CP-51*

Recipient	Donor	Transduction mixture: Cells	Transduction mixture: Phage	Tet^R colonies/ml
B. thuringiensis 4042B M45 Trp^-	4042B M45 tdl Tet^R	2 ml	0.2 ml (1.6×10^{10})	228
"	4060C M473 tdl Tet^R	2 ml	0.2 ml (1.5×10^{10})	253
"	Weybridge A M18 tdl Tet^R	1 ml	1 ml (4.8×10^9)	295
"	None	2 ml	None	0
B. thuringiensis 4060C M473 Ind^-	4042B M45 tdl Tet^R	2 ml	0.2 ml (1.6×10^{10})	107**
"	4060C M473 tdl Tet^R	2 ml	0.2 ml (1.5×10^{10})	2186**
"	Weybridge A M18 tdl Tet^R	1 ml	1 ml (4.8×10^9)	75**
"	None	2 ml	None	0**
B. anthracis Weybridge A M18 (pyrA)	4042B M45 tdl Tet^R	2 ml	0.2 ml (1.6×10^{10})	0
"	4060C M473 tdl Tet^R	2 ml	0.2 ml (1.5×10^{10})	0
"	Weybridge A M18 tdl Tet^R	1 ml	1 ml (4.8×10^9)	5
"	None	2 ml	None	0

*Transductions were carried out as in Table 2. The tdl Tet^R donors were strains that had previously acquired the pBC16 plasmid by transduction.

**Spontaneous resistant colonies were always obtained when mutants of 4060C were the recipients. However, they appeared on the selection plates several hours later than the transductants that had acquired pBC16.

The data in Tables 2 and 3 were obtained by a procedure in which the transductions were carried out in shaken tubes. After a period of one or two hours phage antiserum was added to prevent further lysis and the mixtures were incubated an additional one or two hours to allow expression of tetracycline resistance before samples were plated on L agar plates containing tetracycline. This procedure worked better with the strains of B. thuringiensis than with B. anthracis. The recovery of Tet^R transductants of the Weybridge strain was improved greatly by the use of Millipore membranes as shown in Table 4. Cells and phage were spread together on membranes placed on L agar plates without tetracycline. After a period of incubation at 37°C (5 to 7 hours) the membranes were transferred to L agar plates containing tetracycline (10 to 25 µg/ml). The reason for the low frequency of transduction obtained with B. anthracis by the tube method has not been determined. However, it is presumably a reflection of slow adsorption of CP-51 to B. anthracis cells or delayed phenotypic expression of tetracycline resistance in that organism.

Some of our tests indicated that more transductants were obtained with the Weybridge strain when concentrations of tetracycline lower than 25 µg/ml were used for selection, i.e., 10 to 15 µg/ml. Regardless of the concentration of tetracycline used for selection of Tet^R transductants, the isolated transductants were fully resistant, i.e., they grew in the presence of 50 to 100 µg/ml. Twenty five to 50 µg of tetracycline per ml were used routinely when it was desirable to grow the Tet^R strains in the presence of the antibiotic.

Some of the data from the plasmid transduction experiments suggest that restriction may occur in some of the crosses. For instance, when B. cereus GP7 was the donor, the efficiency of transduction was very low, suggesting that DNA from GP7 may be restricted in the recipients. Data in Table 3 suggest that DNA from B. thuringiensis 4060C may be restricted in B. thuringiensis 4042B and the Weybridge strain of B. anthracis. However, further quantitative experiments would need to be done to confirm that restriction does, in fact, occur. The present data are too limited to be conclusive.

Table 4

Transduction of B. anthracis to tetracycline resistance on Millipore membranes*

Donor	Recipient	Plating mixture		Transductants per membrane
		Cells/0.1 ml	Phage/0.1 ml	
B. thuringiensis 4060C M473 tdl Tet^R	Weybridge A M18 (pyrA)	4×10^7	7×10^9	20
None	"	4×10^7	0	0
Weybridge A M18 tdl Tet^R	Weybridge A M18 td2 Cured 25**	4×10^7	1×10^9	142
None	"	4×10^7	0	0

*Cells and phage (0.1 ml of each) were spread together on Millipore HA membranes placed on L agar. Plates were incubated at 37°C for 5 hours and the membranes were then transferred to L agar plates containing 25 µg of tetracycline per ml. Incubation was continued for 36 hours. Recipients were grown as described in the footnote to Table 2.

**Weybridge A M18 td2 Cured 25 was cured of the Tet^R plasmid, pBC16, and the B. anthracis toxin plasmid simultaneously.

A point of interest observed in the transduction experiments is that although spontaneous Tet^R mutants were usually found in large numbers when B. thuringiensis strain 4060C was the recipient, none has thus far been found with B. thuringiensis 4042B or B. anthracis. When spontaneous resistant mutants were observed with strain 4060C they always appeared on the plates several hours later than the Tet^R transductants that had acquired plasmid pBC16.

(3) Isolation of mutants. The procedure we are using for isolation of mutants of the Weybridge strain was outlined in the Annual Report, December 1980. Because minimal 0 medium contains only five amino acids (in addition to glutamic acid), only one vitamin, and no purines or pyrimidines, and minimal M contains only two amino acids in addition to glutamic acid, these two media are very useful for the isolation of a wide variety of auxotrophic mutants. The mutants we are using for chromosomal mapping and those we use when we need marked strains were isolated with either minimal 0 or minimal M as the minimal medium. The mutants we have thus far characterized are listed in Table 5. We are continuing to isolate and characterize new mutants. At the time of writing this report we have about 75 mutants awaiting characterization of their phenotypes.

Table 5

Auxotrophic mutants of B. anthracis Weybridge A strain

Mutant Number	Growth requirement
Weybridge A M2	Tryptophan
M3	Valine
M4	Nicotinic acid
M6	Leucine plus valine
M11	Leucine
M14	Phenylalanine
M17	Purine
M18	Arginine plus uracil
M22	Arginine plus uracil
M23	Uracil
M27	Arginine plus uracil
M31	Purine

(4) Preparation of protoplasts. We have tried several methods for obtaining protoplasts of cells of the Weybridge strain. The most satisfactory method we have devised is the following:

A culture is grown for 15 to 16 hours from a small inoculum of spores in 25 ml of BHI in a 250-ml flask on a shaker (250 rev/min) at 37°C. Two-tenths ml is transferred to 25 ml of BHI broth and this is incubated with shaking at 37°C for three hours. (It is essential to have very young, nonsporulating cells. Generally Weybridge cells do not sporulate well in BHI broth, and that is the reason we chose that medium. However, some mutants do sporulate reasonably well in it and for such mutants we include 0.5% (w/v) of glycerol in the broth. We learned several years ago that the addition of glycerol to growth medium prevents or delays sporulation of B. licheniformis, and we now find similar results with B. anthracis).

Cells from the three-hour culture are collected by centrifuging in a 50-ml tube for 10 minutes at 7,000 rev/min (SS-34 rotor in a Servall centrifuge). The supernatant fluid is decanted and cells from one to three flasks of culture are resuspended in 5 ml of minimal I medium containing 25% sucrose (glucose and $FeCl_3$ omitted) at pH 8.0. The resuspended cells are incubated statically at room temperature for 16 to 20 hours. Examination with the phase microscope shows that usually 90% or more of the cells are converted to protoplasts.

We use protoplasts prepared in this manner for isolation of plasmid and chromosomal DNA, and we have also used them in fusion experiments with B. subtilis protoplasts (described below). Thus far we are not able to regenerate Weybridge protoplasts reproducibly. We have achieved regeneration in a few experiments, but we can not regenerate them routinely. We will persist in our efforts to regenerate them routinely as we feel they will be useful in transformation experiments to carry out genetic engineering types of procedures.

(5) Fusion of B. subtilis and B. anthracis protoplasts. Protoplasts, prepared as described above, of Weybridge A M18 (pBC16) pyrA Tet^R and protoplasts of B. subtilis 168 Trp^- Str^R, prepared by treatment with lysozyme, were mixed together in the presence of polyethyleneglycol for a few minutes to bring about fusion. The polyethyleneglycol was removed and the protoplasts were incubated in a rich medium for 90 minutes to allow phenotypic expression of tetracycline resistance. They were then plated on regeneration medium (12) containing tetracycline and streptomycin. The streptomycin was added to select against B. anthracis and tetracycline was added to select cells regenerated from protoplasts that had picked up the pBC16 plasmid from B. anthracis by fusion. (The fusion was done in the presence of deoxyribonuclease to prevent transformation).

A number of Tet^R Str^R colonies were obtained and they were shown to have retained the trp mutation, thus rendering the possibility of contamination unlikely. The few "fusons" (regenerated fusion product) that have been screened for plasmids have been shown to contain the pBC16 plasmid. We plan to screen more fusons to see whether the B. anthracis toxin plasmid can be found in them. It seems reasonable to expect that co-transfer should occur and that both plasmids should be found in some of the fusons.

IV. Physiological and metabolic factors affecting protective antigen synthesis and accumulation.

We have not done extensive tests on growth conditions affecting protective antigen yields. We have operated under the idea that investigation of B. anthracis genetics is likely to be more fruitful in eventually improving protective antigen and toxin yields than extensive tests on variations in medium and culture conditions. We have done tests on protective antigen production to confirm that we can obtain reproducible results routinely and that we have a good system for testing various mutants and/or variants resulting from genetic manipulations.

We find that yields of protective antigen are somewhat better in the casamino acids medium of Thorne and Belton (1) with increased amounts of bicarbonate and glucose (13) than in a completely synthetic medium. With respect to antigen production in a completely synthetic medium, the 599 medium of Wright, et al (14) as modified by Thorne and Belton (1) is considerably better than the 1095 medium (15).

Table 6 reports the yields of protective antigen produced by wild-type Weybridge and some mutants isolated from it.

Table 6

Protective antigen production in casamino acids medium

Strain	Reciprocal of highest dilution giving a line in agar diffusion test*
Weybridge (wild type)	16
Weybridge A	16
Weybridge B	16
Weybridge M44A Trp^-	8
Weybridge M44B Trp^-	16
Weybridge M44-1 Trp^- Spo^-	16**
Weybridge A M2 Trp^-	8
Weybridge A M4 Nic^-	16***
Weybridge A M11 Leu^-	16
Weybridge A M18 *pyrA*	24
Weybridge A M23 Ura^-	16
V770	16

FOOTNOTES TO TABLE 6

*The antiserum was from the second bleeding of horse RB (March 4, 1981). The agar diffusion method was as described by Thorne and Belton (1). All positive filtrates produced at least two lines of precipitation. Separation of the lines was more effective when the antiserum was diluted 1 to 2 or 1 to 4, although with diluted antiserum the lines were less intense.

**I originally reported that this mutant produced no detectable protective antigen (Annual Report, December 1980). However, in more recent tests normal yields were produced. I have no explanation for the earlier result.

***I originally reported a titer of 1 for this mutant. However, that was in medium supplemented with nicotinamide. The mutant produced a normal yield when the medium was supplemented with nicotinic acid instead of nicotinamide.

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